

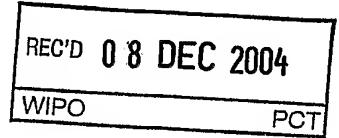


Europäisches
Patentamt

European
Patent Office

Office européen
des brevets

PCT/IB04/4022



Bescheinigung

Certificate

Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. Patent application No. Demande de brevet n°

03293185.9

**PRIORITY
DOCUMENT**

SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk





Anmeldung Nr:
Application no.: 03293185.9
Demande no:

Anmeldetag:
Date of filing: 16.12.03
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Bio-Rad Pasteur
3, boulevard Raymond Poincaré
92430 Marnes-la-Coquette
FRANCE

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Oligonucleotides for the detection of hepatitis B virus

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12Q1/70

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PT RO SE SI SK TR LI



Oligonucleotides for the detection of hepatitis B virus.

The invention relates to oligonucleotide primers and probes for rapid and sensitive detection of hepatitis B virus by amplification, and to methods using the 5 same.

Hepatitis B virus (HBV) is a member of the hepadnavirus family. These are double-stranded DNA viruses which replicate, unusually, by reverse transcription. A number of variants of this virus have been described. HBV is a major causative agent of chronic hepatitis and has been implicated in liver cirrhosis and 10 hepatocellular carcinoma. Hepatitis B virus is endemic in the human population and hyperendemic in many parts of the world. It is estimated that more than one third of the world's population has been infected with the hepatitis B virus. About 5% of the population are chronic carriers of HBV, and nearly 25% of all carriers develop serious liver diseases such as chronic hepatitis, cirrhosis, and primary hepatocellular 15 carcinoma. HBV infection causes more than one million deaths every year.

Antibody and cell-mediated immune responses to various types of antigens are induced during the infection. The immune response to infection with hepatitis B virus is directed toward at least three antigens: hepatitis B surface antigen, the core antigen (HBcAg), and the e antigen (HBeAg). The surface antigen appears in the sera of most patients during the late stage of the incubation period, 20 i.e. 2-8 weeks post-infection, and persists during the acute illness and sharply decreases when antibody to the surface antigen becomes detectable. IgM to the core antigen is found in the serum 2-10 weeks after the surface antigen appears. It correlates with the amount and duration of virus replication. As to Hepatitis B e 25 antigen, it is secreted by infected hepatocytes but eventually, in most individuals, seroconversion from HBeAg to anti-HBe is observed, associated with the immune clearance of infected hepatocytes.

Immunoassays have been developed to detect and measure HBV antigens and antibodies.

30 However, such immunoassays may generate false negative results. Indeed, where an immunological method is carried out within the seroconversion phase of the subject, HBV infection may remain undetected as none circulating antigen or antibodies would be found. Accordingly, diagnostic methods relying on the

detection of HBV nucleic acid provide a more accurate method allowing for an earlier detection of the virus.

Several diagnostic kits have been marketed in this field, for example, 5 Amplicor HBV Monitor and Cobas Amplicor HBV Monitor (Roche Molecular Systems), Versant HBV DNA (bDNA, Bayer Diagnostics) and Digene Hybrid-Capture 2 HBV DNA test (Digene Corporation). However, these tests have generally a limited range of quantification with no more than 4 log: 10^3 to $4 \cdot 10^6$ copies/ml for Amplicor HBV Roche ($2 \cdot 10^2$ to $2 \cdot 10^5$ copies/ml for Cobas HBV Roche), $7 \cdot 10^5$ to $5 \cdot 10^9$ 10 copies/ml for Versant HBV Bayer and $7 \cdot 10^5$ to $5.6 \cdot 10^9$ copies/ml for Digene HBV DNA ($4.7 \cdot 10^3$ to $5.7 \cdot 10^7$ copies/ml for Ultrasensitive Digene HBV DNA). Thus, the main disadvantage of all of these tests resides in the fact that none of them makes it possible to clearly and simply quantify HBV from very low to very high concentrations. Therefore, it is indeed often necessary to combine at least two tests 15 in order to obtain an accurate result, particularly in the case of low or high viral concentration level.

Thus, there is a need for accurate quantification of HBV in a large range, whatever the concentration of the virus in a sample and without the need to perform any additional test.

20 In addition there is still a need for a very sensitive quantitative test that specifically detects all HBV genotypes known in the art.

In this context, the inventors have designed primers and probe that provide particularly rapid and sensitive means for detecting HBV nucleic acid from genotypes A to G with a very wide range of quantification.

25

Definitions

In accordance with the present invention there may be employed any conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, 30 e.g., Sambrook et al., 1989 ; DNA Cloning: A Practical Approach, Volumes I and II (D.N. Glover ed. 1985) ; Oligonucleotide Synthesis (M.J. Gait ed. 1984) ; Nucleic Acid Hybridization [B.D. Hames & S.J. Higgins eds. (1985)] ; Transcription and Translation [B.D. Hames & S.J. Higgins, eds. (1984)] ; Animal Cell Culture [R.I.

Freshney, ed. (1986)] ; Immobilized Cells and Enzymes [IRL Press, (1986)] ; B. Perbal, 1984 ; F.M. Ausubel et al. (eds.), 1994.

5 A "nucleic acid molecule" refers to any nucleic acid : it may be synthetic or not, recombinant or naturally occurring, linear or circular. It may be either in single stranded or in double stranded form. These nucleic acid molecules include genomic DNA, cDNA or RNA. Unless otherwise specified, sequences are described according to the normal convention of giving only the sequence in the 5' to 3' direction along the non-transcribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

10 As used herein, the term "oligonucleotide" refers to a nucleic acid sequence, which can be used as primer in an amplification procedure or as probe in a method of detection. In the context of the invention, these oligonucleotides consists of at least 10, preferably at least 15, and more preferably at least 20 nucleotides, preferably no more than 100 nucleotides; more preferably no more than 40
15 nucleotides that are hybridizable to a genomic DNA molecule, a cDNA molecule, or a mRNA molecule encoding a gene, mRNA, cDNA, or other nucleic acid of interest.

20 A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA, when a single stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule under the appropriate conditions of temperature and solution ionic strength (see Sambrook et al., 1989). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology
25 between two nucleotide sequences, the greater the value of Tm for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher Tm) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating Tm have been derived (see Sambrook et al., 1989, 9.50-9.51). For
30 hybridization with shorter nucleic acids; i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., 1989, II.7-11.8). A minimum length for a hybridizable nucleic acid is at least about 10 nucleotides, preferably at least about 15 nucleotides, and more preferably the length is at least about 20 nucleotides.

PCR is performed under high stringency conditions, by using an annealing temperature of at least 50°C in a high ionic strength buffer. According to a preferred embodiment of the invention, the temperature chosen for the annealing step ranges from 55°C to 60°C and the ionic strength is obtained by using a combination of KCl and MgCl₂ which promotes a high ratio of specific to non specific oligonucleotide binding during the annealing step of each PCR cycle. This enables for stringent primer annealing conditions, leading to increased PCR specificity. For instance, a combination of KCl 50 mM and MgCl₂ 6 mM may be appropriate to confer high specificity. However, other ionic strength conditions may be used and can be readily determined by the one skilled in the art.

"Amplification" of DNA, as used herein, denotes the increase in the concentration of a particular DNA sequence within a mixture of DNA sequences. The amplification step may be carried out by any method using conventional methods of enzymatic amplification of DNA or RNA, such as in particular the TAS (Transcription-based Amplification System) technique proposed by Kwok et al. (1989), the 3SR (Self-Sustained Sequence Replication) technique described by Fahy et al. (1991), the NASBA (Nucleic Acid Sequence-Based Amplification) technique described in patent EP 329 822, or alternatively the SDA (Strand Displacement Amplification) technique described by Walker et al. (1992), or the Ligase Chain Reaction (LCR) technique described in European patent EP 0 320 308, or the Transcription Mediated Amplification (TMA) described in US 5,399,491, or advantageously the PCR technique as described by Saiki et al. (1988) and in European patents EP 0 200 362 and EP 0 201 184, or alternatively the techniques derived from the latter and any other method desired for amplifying nucleic sequences in vitro.

The use of polymerase chain reaction (PCR) is more particularly contemplated in the context of the invention.

As used herein, the terms "primer" and "probe" refer to the function of the oligonucleotide. A primer is an oligonucleotide used for amplifying a target sequence typically by extension of the oligonucleotide after hybridization to the target sequence or by ligation of multiple oligonucleotides which are adjacent when hybridized to the target sequence. A probe oligonucleotide is used to capture or detect a target sequence to which it hybridizes. However the same oligonucleotide probe may also function as a primer. It will therefore be appreciated that any of the sequences disclosed herein for amplification, detection or quantitation of HBV may be used

either as hybridization probes or as amplification primers for detection or amplification.

The term "*hepatitis B virus*" or "*HBV*" denotes the type species of the genus Orthohepadnavirus, family Hepadnaviridae. HBV nucleic acid is a partially double stranded and single stranded circular DNA. The total genome length is about 5 3020-3320 nucleotides (nt) (for the full length strand), or 1700-2800 nt (for the short length strand). As used herein, HBV is meant for any subtype, strain, or variant, e.g. subtype *ad*, *adr*, *adw*, *adyw*, *ar*, *ayw*. In the context of the invention, nucleotide positions are indicated by reference to the HBV genome sequence, subtype *adw*, 10 deposited at GenBank under accession number X98077 (SEQ ID NO:1).

The term "*biological sample*" refers to any body fluid, such as urine, sang, serum, plasma, LCR or tissues like liver biopsy...

Probes and primers of the invention

15 The inventors have demonstrated that a particularly rapid and sensitive detection of HBV infection can be achieved by amplifying a HBV nucleic acid using the specific primers and probe as defined below.

20 These primers and probes have been designed to hybridize to an overlapping region of HBV genome, *i.e.* a region that includes the end of the HBV DNA polymerase encoding region (nucleotides (nt) 2307-3215/1-1623 of HBV circularised genome) and the beginning of the sequence encoding the HBV X protein (nt 1374-1838). These primers and probe allow the amplification and the detection of a 162 nucleotide fragment of the HBV genome (region spanning from nt 1440 to 1602 of SEQ ID NO:1).

25 The detection tests developed herein prove to be of high interest for viral load testing of HBV patients or for the safety screening of blood products.

30 The invention thus provides an oligonucleotide that includes sequence 5' GCTGAATCCCCGCGGACGA 3', (SEQ ID NO:2) or its complementary sequence. This oligonucleotide is particularly useful as a sense primer for amplifying a HBV nucleic acid.

The invention also provides an oligonucleotide that includes a sequence selected from the group consisting of the sequence 5' GTGCAGAGGTGAAGCGAAGTG 3' (SEQ ID NO:3), the sequence 5' GTTCACGGTGGTCGCCATG 3' (SEQ ID NO:4), the sequence

5' GTTCACGGTGGTCTCCATG 3' (SEQ ID NO:5), the sequence
 5' CGTTCACGGTGGTCGCCATGC 3' (SEQ ID NO:6), and the sequence
 5' CGTTCACGGTGGTCTCCATGC 3' (SEQ ID NO:7), as well as their
 complementary sequence. These oligonucleotides are particularly useful as an
 5 antisense primer for amplifying a HBV nucleic acid.

Furthermore, the invention also provides an oligonucleotide, useful as a probe, which includes a HBV binding sequence consisting of an antisense sequence selected from the group consisting of the sequence
 5' GGAGTCCCGCGTAAAGAGAGAGGTG 3' (SEQ ID NO:8), the sequence
 10 5' GGAGACCGCGTAAAGAGAGAGGTG 3' (SEQ ID NO:9), the sequence
 5' GGAGTCTGCGTAAAGAGAGAGGTG 3' (SEQ ID NO:10), and the sequence
 5' GGAGACTGCGTAAAGAGAGAGGTG 3' (SEQ ID NO:11), as well as their complementary sequence.

The oligonucleotides of the invention may possibly comprise additional
 15 sequences linked to the 5' and/or 3' terminus of above-designed sequences, and
 which are foreign to the desired sequence, such as a labelling molecule. Said
 oligonucleotides are nevertheless capable of hybridizing under high stringency
 conditions with the complementary nucleic sequences present in HBV, and said
 additional sequences are unable to hybridize with HBV nucleic acid under standard
 20 hybridization conditions.

These additional sequences may serve as a spacer, linker, or sequence
 for labelling or binding of an enzyme, etc.

The oligonucleotide of the invention especially the probe that includes a
 sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID
 25 NO:10, SEQ ID NO:11, and their complementary sequence, may be detectably
 labelled.

Standard labelling agents (e.g. enzyme, radioactive, or fluorescent
 moieties) may be used for that purpose.

Labelling of the probe is particularly advantageous to facilitate the
 30 detection of the amplified nucleic acid, during a "real-time" amplification/detection
 reaction, i.e. during a PCR process wherein the target sequence is detected and/or
 quantified while the amplification reaction is occurring.

Such detection may be achieved for instance using the nucleic acid
 Molecular Beacon technology (Tyagi and Kramer, 1996; Cayouette et al., 1999).

According to the Molecular Beacon technology, one of either a fluorophore or quencher moiety is attached to each termini of the probing sequence. In the absence of the target nucleic acid, the arm sequences anneal to each other to thereby form a loop and hairpin stem structure which brings the fluorophore and quencher together.

5 When contacted with target nucleic acid, the complementary probing sequence and target sequence will hybridize. Because the hairpin stem cannot coexist with the rigid double helix that is formed upon hybridization, the resulting conformational change forces the arm sequences apart and causes the fluorophore and quencher to be separated. When the fluorophore and quencher are separated, the fluorescent signal

10 is detectable.

All dyes and quenchers known in the art can be used. According the invention, the dye may be preferably selected from the group consisting of Fam, Tet, Hex, Tamra, Texas Red and Cy5, and the quencher may be preferably selected from the group consisting of Dabcyl, Eclipse Dark Quencher, and Black Hole Quenchers.

15 Such molecules are readily available from Eurogentec, Biosearch Technology, Proligo....

The invention thus also provides an oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence and carries a fluorophore 20 moiety at one terminus, and a quencher moiety at the other terminus. Spacer sequences are preferably introduced to link said moieties to one of the sequences SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, or their complementary sequence, so as to provide an extended probe.

Oligonucleotides consisting of the sequences

25 5' CGGCAGGAGTCCCGTAAAGAGAGAGGTGTGCCG 3' (SEQ ID NO:12),
5' CGGCAGGAGACCGCGTAAAGAGAGAGGTGTGCCG 3' (SEQ ID NO:13),
5' CGGCAGGAGTCTCGTAAAGAGAGAGGTGTGCCG 3' (SEQ ID NO:14), and
5' CGGCAGGAGACTCGTAAAGAGAGAGGTGTGCCG 3' (SEQ ID NO:15) are an examples of such extended probe, and are also part of the present invention. The 30 specific pair of spacer sequences has been designed in view of the particular oligonucleotide sequence to which said spacers were to be attached to, and in order to provide a probe which can form a hairpin loop. Such an extended probe further carrying a fluorophore moiety at one terminus, and a quencher moiety at the other terminus, is useful a molecular beacon probe.

The use of an oligonucleotide as defined above as a probe or primer, for hybridizing with and optionally amplifying a nucleic acid from a hepatitis B virus (HBV) is also within the scope of the invention.

In that respect, the present invention further provides a first set of oligonucleotides, used as primers, and consisting of an oligonucleotide that includes SEQ ID NO:2, and at least an oligonucleotide selected from the group consisting of an oligonucleotide that includes SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7. In particular, said first set of oligonucleotides may consist of an oligonucleotide that includes SEQ ID NO:2, an oligonucleotide that includes SEQ ID NO:3, an oligonucleotide that includes SEQ ID NO:4, an oligonucleotide that includes SEQ ID NO:5, an oligonucleotide that includes SEQ ID NO:6 and an oligonucleotide that includes SEQ ID NO:7. Preferably said first set of oligonucleotide consists of, (i) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:3, or (ii) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:4, or (iii) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:5, or (iv) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:6, or (v) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:7, or (vi) an oligonucleotide that includes SEQ ID NO:2, an oligonucleotide that includes SEQ ID NO:4 and an oligonucleotide that includes SEQ ID NO:5, or (vii) an oligonucleotide that includes SEQ ID NO:2, an oligonucleotide that includes SEQ ID NO:6, and an oligonucleotide that includes SEQ ID NO:7.

Still preferably, said first set of oligonucleotides, used as primers, consists in SEQ ID NO:2 and SEQ ID NO:3 ; SEQ ID NO:2 and SEQ ID NO:4 ; SEQ ID NO:2 and SEQ ID NO:5 ; SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:5 ; SEQ ID NO:2 and SEQ ID NO:6 ; SEQ ID NO:2 and SEQ ID NO:7 ; or SEQ ID NO:2, SEQ ID NO:6 and SEQ ID NO:7.

A further subject of the invention is a second set of oligonucleotides, useful for amplifying and detecting HBV nucleic acid, that comprises :

a) a set of oligonucleotides consisting of (i) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:3, or (ii) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:4, or (iii) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:5; or (iv) an oligonucleotide that includes SEQ ID NO:2, and

an oligonucleotide that includes SEQ ID NO:6, (v) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:7, (vi) an oligonucleotide that includes SEQ ID NO:2, an oligonucleotide that includes SEQ ID NO:4 and an oligonucleotide that includes SEQ ID NO:5, or (vii) an oligonucleotide that includes SEQ ID NO:2, an oligonucleotide that includes SEQ ID NO:6 and an oligonucleotide that includes SEQ ID NO:7;

5

b) an oligonucleotide that includes SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, or their complementary sequence.

The invention further provides a method for specifically detecting a HBV 10 by amplification in a biological sample, which method comprises the steps consisting of:

a) contacting a first set of oligonucleotides, used as primers, as defined above, with a biological sample or a nucleic acid preparation obtained from a biological sample under conditions suitable for the oligonucleotides to hybridize to a 15 HBV nucleic acid present in the sample;

b) amplifying said HBV nucleic acid by polymerase chain reaction using said oligonucleotides as primers;

c) detecting the amplification product, indicative of the presence of a HBV in the biological sample.

20 Preferably, the detection of said amplification product is performed by using an oligonucleotide that includes SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, or their complementary sequence, that is detectably labelled, as a probe. Such probe may be introduced at any step. Conveniently it is present or added to the mixture of primers.

25 The amplification method generally comprises the steps consisting of:

a) hybridizing at least one oligonucleotide primer as defined above to a template DNA consisting of a HBV DNA likely to be present in a biological sample;

b) carrying out a primer extension reaction to give a primer extension product;

30 c) denaturing the resulting DNA duplex to separate the primer extension product from the template HBV DNA; the primer extension product functioning as the other template DNA for the other primer, and

d) repeating a cycle of simultaneous primer extension reaction with two oligonucleotide primers, separation of the primer extension products from the DNA templates, and hybridization of primers to amplify a region of the target DNA.

5 Said biological sample may be of any type, e.g. a biological fluid, such as blood, serum, plasma or a tissue sample, such as obtainable by a liver biopsy. More preferably, the biological sample is serum or plasma.

10 The diagnostic method of the invention may be performed on a nucleic acid (e.g. DNA) preparation obtained from such biological sample, for instance by standard extraction procedures.

The invention further provides a kit for amplifying HBV in a biological sample, which kit comprises :

15 - at least a first set of oligonucleotides according to the invention, useful as primers; and

- means for amplifying a HBV nucleic acid.

Means for amplification may include for instance a thermostable DNA polymerase, dNTP solutions, MgCl₂. Uracil DNA-Glycosylase may further be used to prevent PCR contamination.

20 In addition, the kit may further comprise a negative control (i.e. a sample free from HBV nucleic acid), a positive control (i.e. a double strain nucleic acid sequence of the HBV region to be amplified, cloned or not) and an internal control (i.e. a double strain nucleic acid sequence added to the sample and which detection after amplification can be differentiated from the target).

25 The kit may advantageously further comprises a probe as defined above. For instance, it may comprise a synthetic oligonucleotide that includes SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, or their complementary sequence, detectably labelled and useful as a probe.

30 The present invention will be further illustrated by the following figures and examples.

The one skilled in the art will readily understand that the invention encompasses oligonucleotides harboring sequence modifications as compared to the above described oligonucleotides (i.e. deletion, addition and/or substitution of a

limited number of nucleotides), provided that such modifications are not detrimental to the sensitivity and/or specificity of HBV detection achieved using said oligonucleotide.

5 **FIGURES**

Figure 1 is a diagrammatic representation of pair comparisons of the quantification results provided by the test according to the invention (with SEQ ID N°2 and SEQ ID N°3 as primers and SEQ ID N°12 as probe), by HBV PCR kit Roche, and HBV NGI SuperQuant™ LabCorp. A: invention vs HBV PCR kit Roche; 10 B: invention vs HBV NGI SuperQuant LabCorp; C: HBV NGI SuperQuant LabCorp vs HBV PCR kit Roche.

15 Figure 2 is a diagrammatic representation of the linearity of the detection signal against the number of copies/ml of extracted diluted Accurun™ HBV 325 DNA positive control by the test according to the invention (with SEQ ID N°2 and SEQ ID N°3 as primers and SEQ ID N°12 as probe).

EXAMPLE : Specific detection of HBV by "real-time" PCR

Materials and Methods :

20 1) Samples :

TeraPro™ Hepatitis B genotype Panel (Teragenix, catalog HBVGTP) : 15 positive HBV plasma samples (n° 1 to 15) from 15 different patients of various genotypes (3A, 1B, 5C, 1D, 3E, 2F).

25 TeraPro™ Hepatitis B Worldwide Genotype Panel (Teragenix, catalog HBVGTP-002a) : one positive HBV plasma sample of genotype G (n°50).

All these samples have been genotyped with the Visible Genetics kit (Trugene) and quantified by the HBV DNA kit Roche and the HBV DNA kit NGI (National Genetics Institute) SuperQuant.

30 Quantitation standard curve : use of extracted dilutions of panel Accurun 325 HBV DNA Positive control (BBI) or of sample n°15 from the TeraPro™ Hepatitis B Genotype Panel (Teragenix).

2) Extraction :

DNA was extracted from plasma samples using QiaAmp DNA Blood mini kit, ref Qiagen 51104 (200 µl of sample used) or QIAamp MinElute virus vacuum kit, ref Qiagen 57714 (500 µl of sample used) according to the manufacturer recommendations.

5

3) Amplification :

Real-time PCR was performed using the Platinum quantitative PCR SuperMix UDG (Invitrogen 11730-017) : mix 2X that contains 60 U/ml Platinum Taq DNA Pol, 40 mM tris-HCl pH 8.4, 100 mM KCl, 6 mM MgCl₂, and 400 µM dGTP. To mix 1 X are added 1.5 µM primer SEQ ID N°2, 0.3 µM primer SEQ ID N°3 (or 0.3 µM primer SEQ ID N°4), 0.2 µM molecular beacon Fam-DarkQuencher probe SEQ ID N°12 (Eurogentec) and 3 mM MgCl₂ (to achieve 6 mM MgCl₂). Extracted DNA samples are then added to the mix containing primers, probe and additional MgCl₂.

The steps for the amplification were as follows :

15

- 1 x 2 minutes, 50°C (UDG is acting)

- 1 x 2 minutes, 95°C (UDG is inactivated ; Platinum Taq DNA Pol is activated)

- 50 x (15 seconds, 94°C – 30 seconds, 55°C – 30 seconds, 72°C)

- 4°C

20

4) Interpretation of results

For each assay is determined a threshold cycle (C_t) which is the level of fluorescence that is considered to be significantly above the background level of fluorescence measured in the early cycles of the amplification. A reference standard curve is constructed by plotting the log of known target concentrations against the corresponding C_t. The concentration of an unknown sample is then defined by mapping the corresponding C_t to the standard curve.

Results

30

1) Comparison with existing PCR-based detection methods

14 samples (n° 1 to 14) from the TeraPro™ Hepatitis B genotype panel (Teragenix) diluted in plasma and extracted with the QiaAmp DNA Blood kit were quantified with either the test of the invention (with SEQ ID N°2 and SEQ ID N°3 as primers, and SEQ ID N°12 as molecular beacon probe) or with two existing marketed

PCR-based detection tests : HBV PCR kit from Roche (assay developed to quantify until $2 \cdot 10^9$ copies/ml) and HBV NGI SuperQuant kit from LabCorp (results communicated by Teragenix).

Primers and probe of the HBV PCR kit from Roche are selected in a conserved segment of the precore-core region whereas primers and probe of the HBV NGI SuperQuant kit from LabCorp are selected in a conserved segment of the polymerase gene.

Table 1:

Sample			HBV PCR kit Roche		invention		HBV NGI SuperQuant™ LabCorp	
#	Genotype (Visible Genetics results)	Serotype	# copies/ml	Log # copies/ml	# copies/ml	log # copies/ml	# copies/ml	log # copies/ml
1	A	adw2	$3.95 \cdot 10^8$	8,60	$1,34 \cdot 10^7$	7,12	$3 \cdot 10^8$	8,48
2	A	adw2	$3.1 \cdot 10^4$	4,49	263	2,34	$2,3 \cdot 10^4$	4,36
3	A	adw2	$9.7 \cdot 10^3$	3,99	$1,1 \cdot 10^3$	3,02	$1,1 \cdot 10^3$	3
4	B	adw2	$4.6 \cdot 10^5$	5,66	$1,66 \cdot 10^5$	5,21	$5,5 \cdot 10^5$	5,74
5	E	adw2	$8.7 \cdot 10^3$	3,94	566	2,75	$8,8 \cdot 10^3$	3,94
6	C	adr	$8.5 \cdot 10^8$	8,93	$2 \cdot 10^8$	8,3	$4,9 \cdot 10^8$	8,69
7	C	adr	$1,15 \cdot 10^5$	5,06	399	2,59	$3,1 \cdot 10^3$	3,49
8	C	adr	$6.7 \cdot 10^8$	8,83	$3,15 \cdot 10^8$	8,49	$3,4 \cdot 10^8$	8,53
9	C	ayw2	$1,2 \cdot 10^3$	3,08	494	2,65	10^3	3
10	C	ayw2	$4 \cdot 10^3$	3,60	101	2	$6,9 \cdot 10^3$	3,83
11	D	ayw2	$1,64 \cdot 10^9$	9,21	$1,52 \cdot 10^9$	9,17	$2 \cdot 10^9$	9,3
12	E	ayw4	$7,6 \cdot 10^4$	4,88	$1,01 \cdot 10^4$	4	$9,7 \cdot 10^4$	4,98
13	E	ayw4	$9,7 \cdot 10^3$	3,99	$2 \cdot 10^4$	4,27	10^4	4
14	F	ayw4	$10,6 \cdot 10^3$	4,03	$6,1 \cdot 10^3$	3,82	$7,4 \cdot 10^3$	3,86
50	G	ND	$> 2 \cdot 10^5$ *	> 5,3	$1,66 \cdot 10^7$	7,2	$2,9 \cdot 10^7$	7,46
15	F	ayw4	$1,35 \cdot 10^9$	9,13	Used for calibration		$8,5 \cdot 10^8$	8,92

10

* this sample has been quantified on the Cobas Amplicor HBV Monitor kit with a range of quantification of 200 to $2 \cdot 10^5$ copies/ml

The test according to the invention is able to specifically detect and quantify HBV genotypes A to G.

Overall pair comparison of the results obtained with the three tests indicates that the results provided by the test according to the invention show a good 5 correlation with that provided with each of the HBV PCR kit from Roche, or the HBV NGI SuperQuant™ from LabCorp (figure 1).

2) Sensitivity of detection

a) Linearity of the quantification range

10 To demonstrate that the detection signal (cycle threshold) as obtained with the test of the invention (with SEQ ID N°2 and SEQ ID N°3 as primers, and SEQ ID N°12 as molecular beacon probe) correlates with the number of HBV DNA copies per ml, sample number 15 from the TeraPro™ HBV genotype panel (Teragenix) was diluted in plasma and extracted with the QiaAmp DNA Blood kit. The cycle threshold 15 measured with increasing dilutions of the sample until the detection limit (100 copies/ml) is reported in Table 2 below. The linear relationship between the number of copies and the cycle threshold is illustrated in figure 2.

Table 2

Panel TERAGENIX N°15 copies/ml	Log copies/ml	Threshold Cycle (Ct)			Mean Ct	SD*	CV [¶]	RFU ^{\$}
		1	2	3				
10 ⁹	9	14,7	14,2	14,6	14,5	0,26	1,82%	2750/3000
10 ⁸	8	18,0	17,7	17,7	17,8	0,17	0,97%	2500/3250
10 ⁷	7	20,8	21,0	21,3	21,0	0,25	1,20%	2500/2750
10 ⁶	6	23,4	25,1	24,6	24,4	0,87	3,59%	2250/2400
10 ⁵	5	27,8	27,8	27,6	27,7	0,12	0,42%	2000
10 ⁴	4	30,2	30,4	31,5	30,7	0,70	2,28%	1500/1700
10 ³	3	34,3	35,3	33,8	34,5	0,76	2,22%	1000/1250
100	2	38,0	37,1	37,2	37,4	0,49	1,32%	600/750

20 * Standard deviation

¶ Coefficient of variation in %

\$ Relative Fluorescence Unit

On HBV positive samples extracted with the QiaAmp DNA Blood kit from 25 Qiagen, the test according to the invention is able to quantify HBV from 100 to 10⁹

copies/ml with a good correlation coefficient (0.998) when the log of the copy number is plotted versus the threshold cycle (Ct).

b) Sensitivity of detection

5 The Accurun™ HBV 325 DNA positive control (Boston Biomedica) was used as a reference sample to determine the limit of sensitivity of the test of the invention (with SEQ ID N°2 and SEQ ID N°3 as primers, and SEQ ID N°12 as molecular beacon probe). To that end, the sample was diluted into plasma and DNA was extracted with the QIAamp MinElute extraction kit, as explained above. The 10 results of this analysis, reported in the Table 3 below, show that the test according to the invention made it possible to detect as low as 25 copies/ml of the Accurun™ HBV reference on HBV positive samples extracted with the QIAamp MinElute extraction kit from Qiagen.

15 Table 3:

# copies/ml	Accurun HBV Log # copies/ml	Cycle Threshold (Ct)		mean Ct	SD*	CV [#]	RFU ^{\$}
		1	2				
2000	3,3	31,4	30,6	31,0	0,40	1,29%	500/360
500	2,7	33,5	32,4	33,0	0,55	1,67%	330/375
100	2	35,0	34,5	34,8	0,25	0,72%	250/300
50	1,7	36,6	36,4	36,5	0,10	0,27%	150/175
25	1,4	37,8	37,9	37,9	0,05	0,13%	80/150
10	1	N/A	N/A				0

* Standard deviation

Coefficient of variation in %

\$ Relative Fluorescence Unit

20 The mean limit of sensitivity was further assayed in the same conditions on each of 15 samples from the TeraPro™ HBV genotype panel and on the sample n° 50 from the TeraProTM Hepatitis B Worldwide Genotype Panel as summarized in Table 4.

Table 4

Sample	Genotype (Visible Genetics results)	Cycle threshold	log # copies/ml	Estimated # copies/ml
1	A	36,6	1,66	46
2	A	36,7	1,63	43
3	A	41	0,48	< 25
4	B	34,9	1,69	140
5	E	38,2	1,2	16
6	C	40	0,7	< 25
7	C	39,2	0,9	< 25
8	C	38,4	1,14	14
9	C	38,9	1	< 25
10	C	40,2	0,7	< 25
11	D	36,9	1,58	38
12	E	38,4	1,14	14
13	E	40,1	0,7	< 25
14	F	38,6	1,08	12
15	F	37,9	1,3	20
50	G	37,6	1,3	20
Mean sensitivity limit				< 32

Therefore, the mean sensitivity limit of the test of the invention as obtained with the TeraPro™ HBV genotype panel diluted in plasma and extracted with QiaAmp Min Elute virus vacuum kit was found to be very close to the mean sensitivity limit obtained with the Accurun™ HBV 325 DNA positive control (detection limit < 32 copies /ml compared with 25 copies/ml).

The sensitivity limit of the test according to the invention is improved compared with the PCR-based detection tests on the market, such as the Cobas Amplicor HBV Monitor kit from Roche Diagnostics (sensitivity limit of 200 copies/ml) or the Hepatitis B virus NGI SuperQuant kit from LabCorp (sensitivity limit of 100 copies/ml).

3) Comparison of the set of primers SEQ ID N°2 and SEQ ID N°3 with the set of primers SEQ ID N°2 and SEQ ID N°4

For this comparison, dilutions of Accurun 325 HBV DNA positive control in plasma, extracted with the QiaAmp DNA blood mini kit, were used. Results of 5 threshold cycles (Ct) are reported in table 5.

Table 5 :

Accurun HBV		SEQ ID N°2 + SEQ ID N°3				SEQ ID N°2 + SEQ ID N°4			
# copies/ml	Log # copies/ml	Ct (1)	Ct (2)	Mean Ct	Mean RFUs	Ct (1)	Ct (2)	mean Ct	Mean RFUs
10 ⁵	5	28.9	28.9	28.9	625	27.2	26.0	26.6	550
10 ⁴	4	31.6	31.6	31.6	500	30.0	31.6	30.8	350
10 ³	3	34.3	34.8	34.6	350	33.3	31.9	32.6	225
10 ²	2	37.5	ND	37.5	200	37.8	37.7	37.8	100

10 Both sets of primers amplified the extracted dilutions of Accurun HBV with a same limit of sensitivity (10² copies/ml), but a better reproducibility of threshold cycle and a better correlation were obtained with the set of primers SEQ ID N°2 and SEQ ID N°3 (correlation coefficient of 0.999) than with the set of primers SEQ ID N°2 and SEQ ID N°4 (correlation coefficient of 0.970).

REFERENCES

- Ausubel et al. (eds.), *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994)
- 5 - Cayouette M, Sucharzuk A, Moores J, Tyagi S, and Kramer FR (1999) Using molecular beacons to monitor PCR product formation. *Strategies News*. 12:85-88.
 - Fahy et al. (1991) *PCR Meth. Appl.*, 1, 25-33
 - Kwoh et al. (1989) *PNAS*, 86, 1173-1177
- 10 - Perbal, *A Practical Guide To Molecular Cloning* (1984).
- Saiki et al., (1988), *Science*, 239:487
- Sambrook, Fritsch & Maniatis, *Molecular Cloning: A Laboratory Manual*, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
- 15 - Tyagi S and Kramer FR (1996) *Nature Biotechnol.*, 16, 303-308
- Walker et al. (1992) *P.N.A.S.*, 89, 392-396
- Yaron et al., (1979) *Analytical Biochemistry* 95: 228-235

CLAIMS

1. An oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7 and their complementary sequences.
2. The oligonucleotide according to claim 1, which consists of SEQ ID NO: 2 or its complementary sequence.
3. The oligonucleotide according to claim 1, which consists of SEQ ID NO: 3; or its complementary sequence.
4. The oligonucleotide according to claim 1, which consists of SEQ ID NO: 4, or its complementary sequence.
5. The oligonucleotide according to claim 1, which consists of SEQ ID NO: 5, or its complementary sequence.
6. The oligonucleotide according to claim 1, which consists of SEQ ID NO: 6, or its complementary sequence.
7. The oligonucleotide according to claim 1, which consists of SEQ ID NO: 7, or its complementary sequence.
8. Use of an oligonucleotide as defined in any of claims 1 to 7, as a probe or primer, for hybridizing with and optionally amplifying a nucleic acid from a hepatitis B virus (HBV).
9. Use of an oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence, as a probe for hybridizing with a nucleic acid from HBV.

10. The use according to claim 9, wherein said oligonucleotide consists of a sequence selected from the group consisting of SEQ ID NO:8; SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence.

5 11. The use according to claim 9, wherein said oligonucleotide includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence and carries a fluorophore moiety at one terminus, and a quencher moiety at the other terminus.

10 12. The use according to claim 11, wherein said oligonucleotide consists of a sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 and SEQ ID NO:15, and carries a fluorophore moiety at one terminus, and a quencher moiety at the other terminus.

15 13. A set of oligonucleotides consisting of an oligonucleotide that includes SEQ ID NO:2, and at least an oligonucleotide selected from the group consisting of an oligonucleotide that includes SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7.

20 14. A set of oligonucleotides according to claim 13, which consists of:

(i) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:3;

(ii) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:4;

25 (iii) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:5;

(iv) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:6;

30 (v) an oligonucleotide that includes SEQ ID NO:2, and an oligonucleotide that includes SEQ ID NO:7;

(vi) an oligonucleotide that includes SEQ ID NO:2, an oligonucleotide that includes SEQ ID NO:4 and an oligonucleotide that includes SEQ ID NO:5; and

(vii) an oligonucleotide that includes SEQ ID NO:2, an oligonucleotide that includes SEQ ID NO:6 and an oligonucleotide that includes SEQ ID NO:7.

15. A set of oligonucleotides comprising:

a) a set of oligonucleotides according to claim 13 or 14; and

b) an oligonucleotide that includes a sequence selected from the group

5 consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence.

16. A set of oligonucleotides according to claim 15, that comprises:

a) a set of oligonucleotides according to claim 13 or 14; and

10 b) an oligonucleotide that consists of a sequence selected from the group consisting of SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 and SEQ ID NO:15, and carries a fluorophore moiety at one terminus, and a quencher moiety at the other terminus.

15 17. A method for specifically detecting a HBV by amplification in a biological sample, which method comprises the steps consisting of:

a) contacting a set of oligonucleotides according to claim 13 or 14 with a biological sample or nucleic acid preparation obtained from a biological sample, under conditions suitable for the oligonucleotides to hybridize to a HBV nucleic acid 20 present in the sample;

b) amplifying said HBV nucleic acid using said oligonucleotides as primers;

c) detecting the amplification product, indicative of the presence of a HBV in the biological sample.

25

18. The method according to claim 17, wherein HBV nucleic acid is amplified by polymerase chain reaction.

30 19. The method according to claim 17 or 18, wherein the detection of said amplification product is performed by using an oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence, and that is detectably labelled, as a probe.

20. The method according to claim 19, wherein said oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence, and carries a fluorophore moiety at one terminus, and a quencher moiety at the other terminus.

5

21. The method according to claim 19 or 20, wherein said oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence, is SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 or SEQ ID NO:15.

10

22. A kit for amplifying HBV in a biological sample, which kit comprises :

- at least a set of oligonucleotides according to claim 13 or 14, useful as primers;

15 - means for amplifying a HBV nucleic acid.

23. The kit according to claim 22, which further comprises means for the detection of the amplified product.

20 24. The kit according to claims 22 or 23, wherein the means for amplifying HBV nucleic acid are means for amplification by Polymerase Chain Reaction.

25 25. The kit according to any of claims 22 to 24, which comprises an oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence, detectably labelled and useful as a probe.

30 26. The kit according to claim 25, wherein said oligonucleotide that includes a sequence selected from the group consisting of SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and their complementary sequence, is SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14 or SEQ ID NO:15.

BIO-RAD PASTEUR

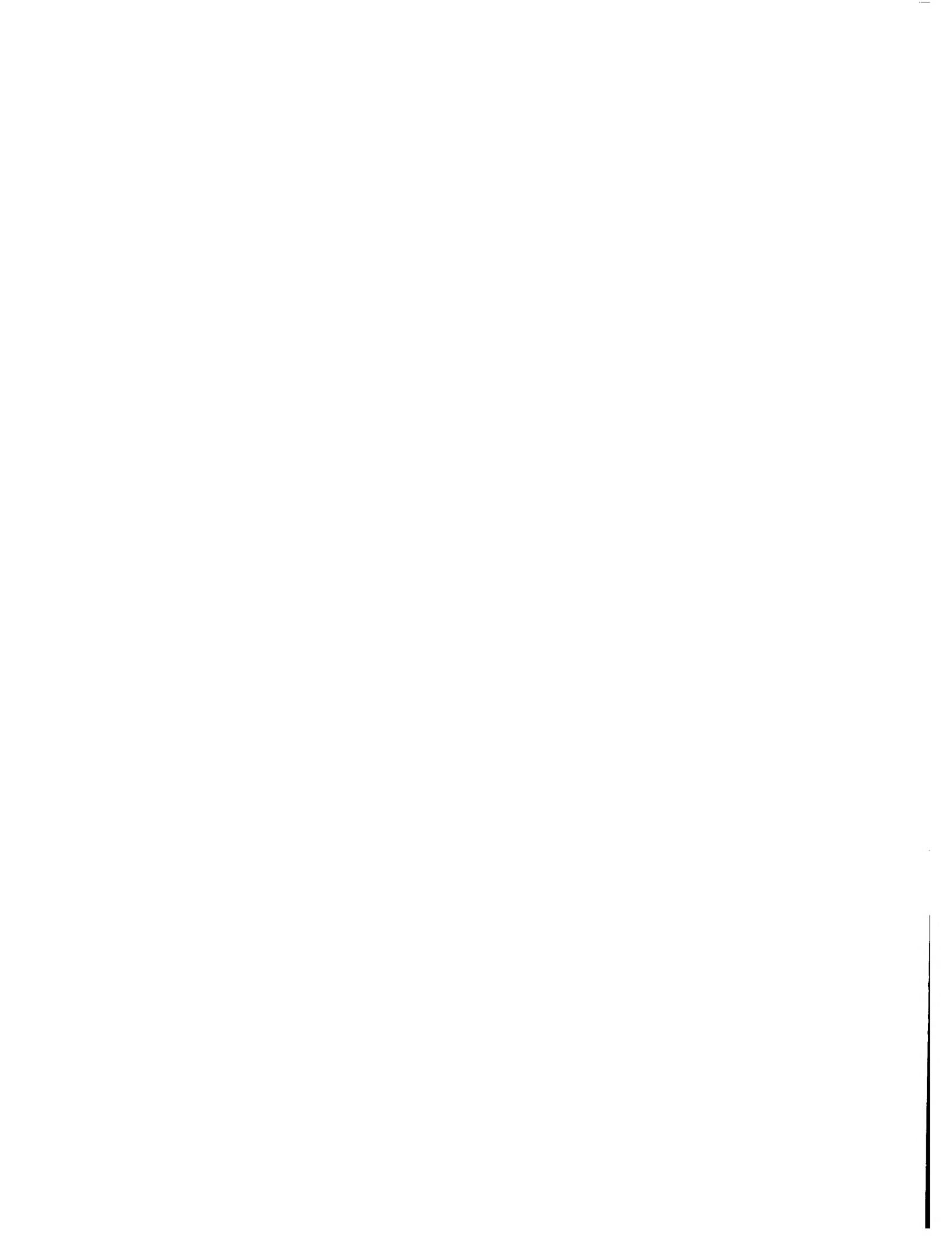
5

10

Abstract of the invention

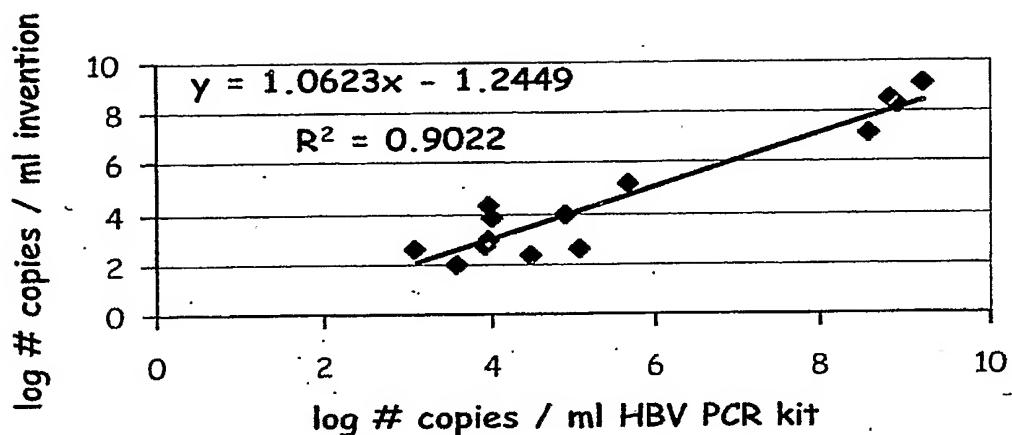
The invention relates to specific oligonucleotide primers and probes for rapid and sensitive detection of hepatitis B virus by amplification of the HBV nucleic acid, 15 and to methods using the same. It is also concerned with kits useful to perform these detection tests.

Figure : none

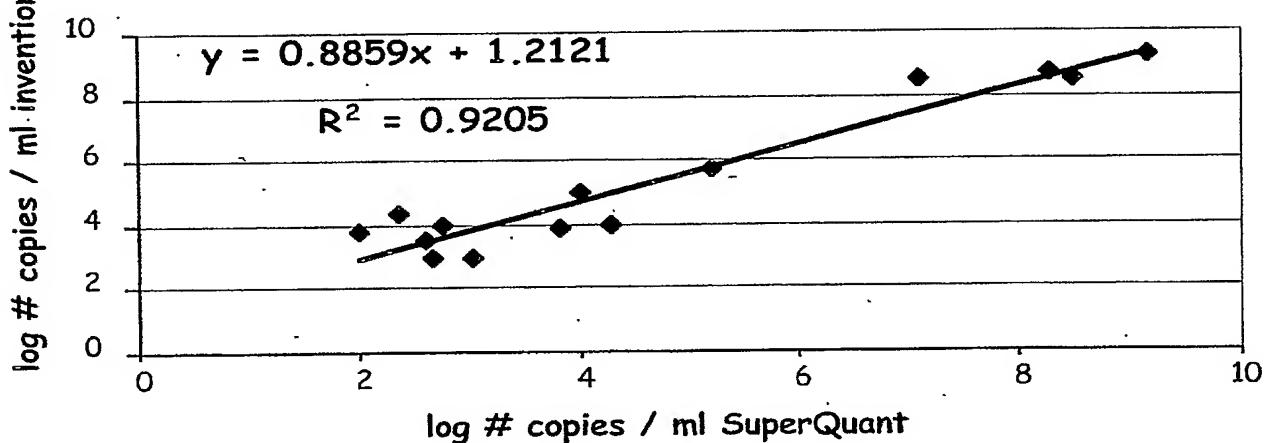


1/2

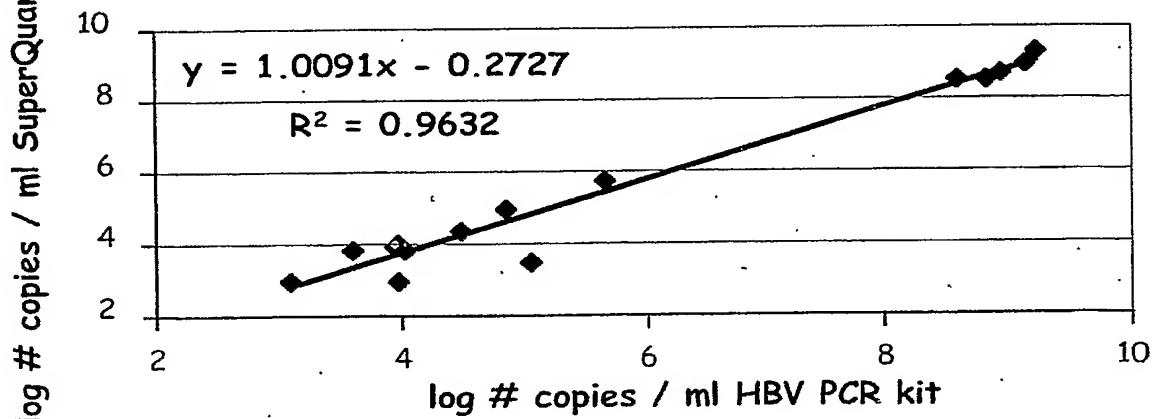
A.



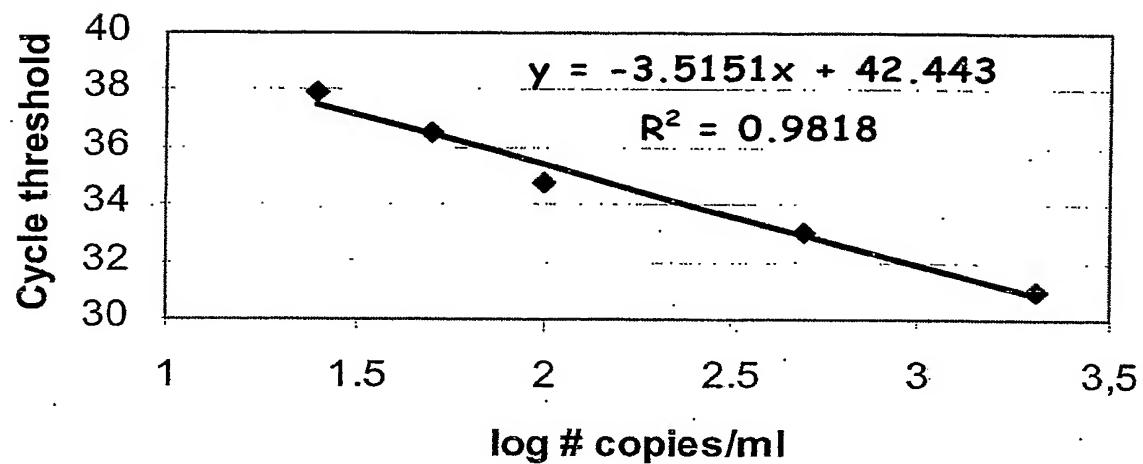
B.



C.

FIG.1

2/2

FIG.2

SEQUENCE LISTING

<110> BIO-RAD Pasteur
<120> Oligonucleotides for the detection of hepatitis B virus
<130> BET 03P0671
<160> 15
<170> PatentIn version 3.1
<210> 1
<211> 3215
<212> DNA
<213> Hepatitis B virus
<400> 1
ctccaccact ttccacccaaa ctcttcaaga tcccagagtc agggccatgt accttcctgc 60
tggtgtggctcc agttcaggaa cagttagccc tgctcagaat actgtctctg ccataatcg 120
aatcttatcg aagactgggg accctgtgcc gaacatggag agcatcgcat caggactcct 180
aggaccctg ctcgtgttac aggccgggtt tttcttgttg acaaaaatcc tcacaataacc 240
acagagtcta gactcgtggt ggacttctct caattttcta gggggAACAC ccgtgtgtct 300
tggccaaaat tcgcagtccc aaatctccag tcactcacca acctgttgtc ctccaaacttg 360
tcctggatcgatgt gtctgcggcg ttttatcatc ttccctctgca tcctgctgtct 420
atgcctcatc ttcttgttgg ttcttctgga ctatcaaggt atgttgcccg tttgtccct 480
aattccagga tcataccacca ccagcacggg accatgcaag acttgcacag ctccctgctca 540
aggaacctct atgtttccct catgttgctg tacaaaacct acggacggaa actgcacctg 600
tattcccatc ccatcatctt gggctttcgc aaaataccta tgggagtgccc cctcagtccg 660
tttctcttgg ctcagtttac tagtgcatt tggcgttg ttcgttagggc tttcccccac 720
tgtctggctt tcagttatgt ggtatgttg gttttgggg ccaagtctgt acaacatctt 780
gagtcccttt ataccgctgt taccaatttt cttttgctt tgggtataaca tttaaacccct 840
cacaaaacaa aaagatgggg atattccctt aacttcatgg gatatgtaat tgggagttgg 900
ggcacattgc cacaggaaca tattgtacaa aaaatcaaaa cgtgttttag gaaacttcct 960
gtaaacaggc ctattgattt gaaagtatgt caacgaattt tgggtctttt ggggtttgcc 1020
gccccctttca cgcaatgtgg atatcctgtt ttaatgcctt tataatgcatt tataacaagca 1080
aaacaggctt ttactttctc gccaacttac aaggccttgc taagtaaaca gtatctgaac 1140
ctttaccccg ttgctcgccca acggccttgc ctgtgccaag tgggtgttgc cgcaacccccc 1200
actgggtggg gcttggccat aggccatcag cgcatgcgtg gaaaccttgc gtctccctg 1260
ccqatccata ctgcggaaact cctagccgct tgggtgttgc gcagcaggc tggggcaaaa 1320

ctcatcgaaa	ctgacaattc	tgtcggtctc	tcccgcaagt	atacatcctt	tccatggctg	1380
ctaggctgtg	ctgccaactg	gatcctgcgc	gggacgtcct	tttttacgt	cccgctggcg	1440
ctgaatcccg	cggaacgaccc	ctcccggggc	cgcttggggc	tctaccggcc	gcttctccgc	1500
ctgttgtacc	gaccgaccac	ggggcgacc	tctctttacg	cgactcccc	gtctgtgcct	1560
tctcatctgc	cggaccgtgt	gcacttcgct	tcacccctgc	acgtcgcatg	gagaccaccg	1620
tgaacgccc	caggaacctg	ccaaaggct	tgcataagag	aactcttgg	ctttcagcaa	1680
tgtcaacgac	cgacccttgag	gcataacttca	aagactgtgt	gtttactgag	tgggaggagt	1740
tgggggagga	ggttaggtta	atgatctttg	tactaggagg	ctgtaggcat	aaattgggtgc	1800
ttcaccaggc	accatgcaac	ttttcacct	ctgcctaatac	atctcttgc	catgtcctac	1860
tgttcaagcc	tccaaagctgt	gccttgggtg	gctttgggac	atggacattg	accgtataa	1920
agaatttgg	gcttctgtgg	agttactctc	tttttgcct	tctgacttct	ttccctgtgt	1980
tcgagatctc	ctcgacacccg	cctctgctct	gtatcggag	gccttagagt	ctccggaaaca	2040
ttgttcaccc	caccatacgg	caatcaggca	agctattctg	tgttgggtg	agttgatgaa	2100
tctagccacc	tgggtggaa	gtaatttgg	agatcaagca	tccaggact	tagtagtcag	2160
ctatgtcaac	gttaatatgg	gcctaaaatt	cagacaacta	ttgtggttc	acatttcctg	2220
tcttacgttt	gggagacaaa	ctgttcttga	atatttgg	tccttggag	tgtggattcg	2280
cactcctcct	gcatatacgc	caccaaattgc	ccctatctta	tcaacacttc	cggaaactac	2340
tgtttaga	caaagaggca	ggacccttag	aagaagaact	ccctcgctc	gcagacgaag	2400
gtctcaatcg	ccgcgtcgca	gaagatctca	atctcgaa	tctcaatgtt	agtattccct	2460
ggacacataa	ggtggaaac	tttactgggc	tttattctc	tacggacact	tgctttaatc	2520
ctaattggca	aactccttct	tttctgaca	ttcatttgca	ggaggacatt	gttgatagat	2580
gtaagcaatt	tgtggggccc	cttacagtaa	atgaaaacag	gagactaaaa	ttaattatgc	2640
ctgcttaggtt	ttatccaaat	gttactaaat	atttgccctt	agataaagg	atcaaaccgt	2700
attatccaga	gtatgttagtt	aatcattact	tccagacgct	acattattta	cacactcttt	2760
ggaaggcggg	gatcttat	aaaagagagt	ccacacgtag	cgccctcattt	tgccggtcac	2820
catattcttgc	ggaacaagat	ctacagcatg	ggaggttgg	cttccaaacc	tgcggaaaggc	2880
atggggacaa	atcttgctgt	ccccaaatccc	ctgggattct	tcccccgtca	tcgttggac	2940
cctgcattca	aagccaaactc	agacaatcca	gattgggacc	tcaacacgca	caaggactac	3000
tggccggacg	catggaaggt	gggagtggga	gcattcgccc	cagggttcac	ccctccccat	3060
gggggactgt	tggggtggag	ccctcaggct	cagggctac	tcacaactgt	gccagcagct	3120

cctcctcctg cctccaccaa tcggcagtca ggaaggcagc ctactccctt atctccacct 3180
 ctaagagaca ctcatccaca ggccatgaag tggaa. 3215

<210> 2
 <211> 18
 <212> DNA
 <213> Artificial : oligonucleotide

<400> 2
 gctgaatccc gcggacga 18

<210> 3
 <211> 21
 <212> DNA
 <213> Artificial : oligonucleotide

<400> 3
 gtgcagaggt gaagcgaagt g 21

<210> 4
 <211> 19
 <212> DNA
 <213> Artificial : oligonucleotide

<400> 4
 gttcacggtg gtcgccatg 19

<210> 5
 <211> 19
 <212> DNA
 <213> Artificial : oligonucleotide

<400> 5
 gttcacggtg gtctccatg 19

<210> 6
 <211> 21
 <212> DNA
 <213> Artificial : oligonucleotide

<400> 6
 cgttcacggc ggtcgccatg c 21

<210> 7
 <211> 21
 <212> DNA
 <213> Artificial : oligonucleotide

<400> 7
 cgttcacggc ggtctccatg c 21

<210> 8
 <211> 22

<212> DNA
<213> Artificial : oligonucleotide

<400> 8
ggagtccgcg taaagagagg tg

22

<210> 9
<211> 22
<212> DNA
<213> Artificial : oligonucleotide

<400> 9
ggagaccgcg taaagagagg tg

22

<210> 10
<211> 22
<212> DNA
<213> Artificial : oligonucleotide

<400> 10
ggagtctgcg taaagagagg tg

22

<210> 11
<211> 22
<212> DNA
<213> Artificial : oligonucleotide

<400> 11
ggagactgcg taaagagagg tg

22

<210> 12
<211> 32
<212> DNA
<213> Artificial : oligonucleotide

<400> 12
cggcaggagt ccgcgtaaag agaggtgtgc cg

32

<210> 13
<211> 32
<212> DNA
<213> Artificial : oligonucleotide

<400> 13
cggcaggaga ccgcgtaaag agaggtgtgc cg

32

<210> 14
<211> 32
<212> DNA
<213> Artificial : oligonucleotide

<400> 14
cggcaggagt ctgcgtaaag agaggtgtgc cg

32

<210> 15
<211> 32
<212> DNA
<213> Artificial : oligonucleotide

<400> 15
cggcaggaga ctgcgtaaag agaggtgtgc cg

32

PCT/IB2004/004022

